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PRINCIPAL INVESTIGATOR: Bruce Line, M.D.

CONTRACTING ORGANIZATION: The Albany Medical College
Albany, New York  12208-3479

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**13. ABSTRACT** *(Maximum 200 words)*

A non-invasive, and accurate imaging technique is needed to further evaluate mammographically suspicious lesions to reduce the number of cancer negative biopsies. We have demonstrated that technetium-99m radiolabeled human alpha-fetoprotein ($^{99m}$Tc AFP) localizes in human breast cancer cells *in-vivo*, most likely concentrating in breast cancer cells due to a specific receptor not found in normal adult breast tissue. Our technical objective is to assess $^{99m}$Tc AFP as an agent to detect and stage human breast cancer. Following the first two aims of the award work scope, we have compared natural full-length and recombinant domain III preparations of $^{99m}$Tc AFP to tracers now used to detect breast cancer, $^{201}$Tl and $^{99m}$Tc sestamibi. Our imaging results in immune-deficient (SCID) mice bearing human cancer xenografts confirm our hypothesis that $^{99m}$Tc AFP is a superior human breast cancer imaging agent. We have also demonstrated that $^{99m}$Tc AFP imaged pulmonary metastases in a rat model of breast cancer. During the extension effort we will continue the work scope for these aims and further determine the ability of $^{99m}$Tc AFP to image metastases from a transplantable rat mammary cancer.

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Bruce R Line MD          7/14/99
PI - Signature                    Date

## Table of Contents

# INTRODUCTION:

Because of the lack of mammographic specificity, most biopsies of the breast are benign. In the US the probability of malignancy when a biopsy is performed on a mammographic abnormality usually ranges from 15-35%.[1-4] Our hypothesis is that AFP is concentrated in breast cancer cells by a receptor-mediated process, thereby providing a novel means for detecting breast cancer cells specifically and with high sensitivity. The work we are carrying out focuses on the development of $^{99m}$Tc alpha-fetoprotein (AFP) as a novel agent to detect and stage breast cancer. To test our hypothesis we pursued the following Specific Aims.

Aim 1. Among the three preparations of human AFP available to us (natural full-length, recombinant full-length, recombinant domain III), establish which one of these consistently yields the highest tumor-to-background ratios in immune-deficient (SCID) mice bearing either ER+ MCF-7 or ER-MDA MB 231 human breast cancer xenografts.

Aim 2. Using the preparation of choice established in Aim 1, determine the breadth of applicability of $^{99m}$Tc AFP as an imaging agent for human breast cancers. This is accomplished by comparing the imaging capability of $^{99m}$Tc AFP to that of $^{201}$Tl and $^{99m}$Tc sestamibi in a broad range os different human tumor xenografts. Tumors consist of benign and malignant human breast tumor lines, non-breast tumor lines and freshly resected patient breast cancers.

Aim 3. Measure the level of AFP receptors in the imaged tumors as a potential explanation for the imaging capability of this protein.

Aim 4. Determine whether $^{99m}$Tc AFP can image metastases of a transplantable rat mammary cancer.

These aims were spread over 7 tasks, of which 1-5 are complete and tasks 6 and 7 are partially complete. Several technical difficulties were experienced in the second year of the project. These issues have been largely resolved. To complete the tasks of this project we have received approval to extend the work for an additional year.

Task 6 seeks to establish AFP receptor content in various breast cancer tumor cell lines. In addition to methodology revisions necessitated by the limited availability of AFP, there have been interuptions in laboratory operations due to reassignment of space that has slowed progress. These issues have been resolved, and we are optimistic that work will progress without further disruption.

Task 7 is work performed in a rat model of breast cancer metastasis to evaluate 99mTc AFP uptake at metastatic sites. Work on this task has been hampered by AFP radiolabeling problems. Our investigations identified the problem which was excessive phosphate buffer concentration in the AFP protein solution. Radiolabeled AFP was thereby reduced with higher fraction of micro-colloid that produced significant alteration of the expected biodistribution of the $^{99m}$Tc AFP. We have resolved these radiolabeling problems and will proceed as previously planned. Recently we have demonstrated that metastatic foci in the lungs of a rat model can be imaged by $^{99m}$Tc AFP (see below).

## BODY OF ANNUAL REPORT

**Research Accomplishments by Task in Scope of Work**

### Task 1: Months 1-4: AFP production.

*Recombinant Domain III AFP:* We produced Domain III of human AFP using a baculovirus vector (pACSecG2T, Pharmingen) which incorporated an N-terminal leader sequence from the bacu-

loviral protein gp67 (to facilitate secretion from insect cells), the Glutathione-S-Transferase (GST) protein from Schistosoma japonicium (to facilitate purification and solubilization of fusion protein) and Domain III of human AFP. The transfer vector containing the coding sequence for Domain III of hAFP was cotransfected into SF9 insect cells to produce recombinant virus. Virus was plaque-purified, then screened for the incorporation of the Domain III coding sequence into the viral genome (by PCR; Polymerase Chain Reaction) and for the ability of recombinant virus to produce secreted protein (Western Blot).

Recombinant baculovirus containing the cDNA for Domain III (previously described) was amplified by three serial passages and titered by plaque assay in order to produce a large quantity of virus. Protein was then produced in large batches by infection of SF9 cells with recombinant virus. The medium containing the secreted Domain III protein is harvested and clarified by centrifugation to remove cell debris which results from cell lysis. The protein is purified by loading the clarified cell culture medium onto a Glutathione-Agrose (Sigma) column. The column was washed with PBS and then treated with Thrombin (Sigma) to release the Domain III fragment. The thrombin released fragment will was identified by Western Blot analysis using polyclonal antibody to human AFP and silver-stained SDS-PAGE. Protein was then aliquoted (50μg/each) and stored lyophilized at -80°C.

*Natural human full length AFP:* HepG2 cells were maintained and grown as a monolayer in αMEM (GIBCO, Grand Island, NY) supplemented with 5% serum (2/5 calf serum, 3/5 fetal calf serum), penicillin G (100 units/ml), and streptomycin (100 μg/ml). Cells were released from monolayer using 0.25% trypsin/0.25% EDTA. Subculturing into additional flasks was carried out by five-fold dilution of cells in the above maintenance medium. Confluent flasks were switched to serum-free medium to up-regulate production of AFP as described by Tecce et al. Serum-free medium is comprised of 3 parts αMEM: 1 part Waymouth's MB 752/1 plus $3 \times 10^{-8}$ M sodium selenite, 2 mM L-glutamine and 1.5% antibiotic/antimycotic mixture from GIBCO. Cells were refed with serum-free medium every three days.

To purify AFP, HepG2 culture supernatants were pooled and concentrated using P-10 Centriprep concentrators (Amicon, Beverly, MA). Ten ml of concentrate containing approximately 3 mg of AFP were loaded onto an 18 cm x 2.5 cm immunoaffinity column (rabbit anti-human AFP (DAKO) conjugated to cyanogen bromide-activated Sepharose 4B) in a loading buffer of 100 mM NaCl/10 mM sodium phosphate pH 7.4. Concentrate was incubated on the column at room temperature for 30 minutes. Non-AFP proteins were eluted with approximately 200 ml loading buffer until no protein was detectable in the eluate by UV absorbance (280 nm). AFP is eluted with approximately 200 ml of 1.8 M $MgCl_2$ and dialyzed immediately against excess 10mM sodium phosphate buffer, pH 7.2. This material was washed and concentrated in a buffer comprised of 100 mM sodium chloride-10 mM sodium phosphate, pH 7.2.

This task has been completed for both recombinant Domain III AFP and natural human full length AFP.

## Task 2: Months 1-4: Grow human breast cancer cell lines as xenografts.

MCF-7 and MDA-MB-231 cell lines were expanded into multiple flasks, grown to confluence, and harvested by trypsinization. Cells were converted to solid tumor form by centrifugation into a pellet and exposure of the cell pellet to 15 μl of fibrinogen (50 mg/ml) and 10 μl of thrombin (50 units/ml) for 30 minutes at 37°C. Fibrin clots were cut into pieces approximately 1.5 mm in diameter. Six to eight pieces were loaded into a 16 gauge trocar and were implanted subcutaneously in the region of the brachial lymph node near the front limb of female CB17 SCID mice. Tumors were usually palpable 3 to 4 weeks after implantation and reached a diameter of 1 cm approximately 6 weeks

after implantation. To assess tracer sensitivity, imaging studies were performed when tumors were first palpable, approximately 0.2 cm in diameter, and as they enlarge to 1 cm in size.(5-7)

MCF-7 and MDA-MB-231 cell lines have been expanded, grown, harvested and implanted into SCID mice. The task is completed as proposed. In addition other cell lines have been implanted as xenografts as described in the results and discussion section.

### Task 3: Months 3 & 4: Image MCF-7 and MDA-MB-231 breast cancer xenografts

$^{201}$Tl and $^{99m}$Tc sestamibi are currently in clinical trial as imaging agents for breast cancer. Although early results have shown some promise (8-10), improvements in both sensitivity and specificity are needed. For example, a recent large multicenter trial enrolled 673 patients included 377 women with non-palpable mammographically detected abnormalities. The overall sensitivity and specificity for $^{99m}$Tc sestamibi was 85% and 81% respectively with 72% sensitivity and 86% specificity for non-palpable tumors(11). False positive sestamibi studies have been found in cases of fibrocystic disease and in fibroadenomas. These false positive results may be due to hypercellularity and proliferative changes(12) or to the non-specific "metabolic activity" of these lesions.(9) Furthermore, $^{99m}$Tc sestamibi is eliminated from the cell by the multidrug resistance P-glycoprotein (gp170) which is over expressed in some breast cancers.(13) Finally, $^{201}$Tl and $^{99m}$Tc sestamibi both produce images with variable patterns of normal breast activity that is in part due to significant scatter from non-specific uptake in cardiac and abdominal tissues.(14)

To evaluate the potential of $^{99m}$Tc-AFP in imaging of breast cancer, each of the AFP preparations was labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice. Mice were imaged and the $^{99m}$Tc AFP preparations were assessed by their resultant tumor to background ratios (T/B) and clearance kinetics (% of injected dose/gram).

$^{99m}$Tc labeling of AFP was carried out via stannous ion reduction as previously described (15). Briefly, Tc99m AFP was prepared from a 50μg AFP aliquot mixed with .5ml 0.9% Sodium Chloride Injection (Baxter Healthcare). The solution was added to an Ultra Tag RBC™ Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO), and the contents of the vial were mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation time, 20-1000 MBq $^{99m}$Tc Sodium Pertechnetate Injection (Mallinckrodt Medical, Inc., St. Louis, MO) was added in a volume of 1-2 ml. The contents of the vial were mixed by gentle swirling and were incubated for 15 minutes. Dose aliquots were assayed using thin-layer chromatography performed on preparation using ITLC-SG (Gelman Instrument Co., Ann Arbor, MI) with acetone. Typically, 92 - 100% of the $^{99m}$Tc was bound to AFP. The preparation was not used in studies if the percent bound was less than 90%.

*In vivo* biodistribution data was collected in up to 6 mice imaged simultaneously on a Siemens gamma camera. The data was collected by a dedicated computer and transferred to a Pentium PC MS Windows-based image processing system for analysis.(16,17) After sedation with intraperitoneal administration of 50 mg/kg pentobarbital, the mice were injected intravenously with 20-40 MBq of the tracer compound ($^{99m}$Tc AFP (3μg), $^{99m}$Tc Sestamibi, or $^{201}$Tl) and then were placed in the prone position on a thin polyethylene panel. To eliminate motion during imaging, the mice were restrained on panels by strips of tape over their extremities so as not to restrict respiration. Dynamic images obtained over 60 minutes were used to determine the biodistribution of the labeled agent. Typically, twelve sequential, five minute images were obtained with low energy general purpose collimation, and 1.5 hardware zoom into computer matrices having 128 by 128 picture elements.(16) Images were analyzed by positioning "regions of interest" (ROI) over the tumor, thigh, contralateral chest wall and total body. The counts were corrected for radioisotope decay to facilitate activity comparisons.

The image data were evaluated to determine the activity in the tumor tissue by drawing a region of interest that included all margins of the tumor. Where the tumors were not visibly apparent, regions were placed over the area where the tumor was palpated at the time of imaging. A second region was placed over the contralateral chest wall by reflecting the tumor region of interest through the midline axis. The second region was used to define a background activity that was subtracted from the tumor region to yield counts in the tumor tissue. The gram weight of the tumor was determined from a volume calculation based on measured tumor diameter. A third region of interest was defined over the thigh and the counts per gram of thigh tissue was obtained by using a thigh mass of 6% of body weight. A fourth region of interest was defined over the entire animal to determine the total injected activity. Regions were produced for each animal study at both 1 and 24 hour image collection points. Tracer localization parameters were determined by 1) the tumor uptake as the percent of injected activity per gram of tissue (%ID/gram); and 2) the tumor to background (TB) ratio determined from the ratio of tumor activity per gram to the thigh activity per gram.

Each of the AFP preparations (recombinant Domain III of human AFP and natural full length AFP) was labeled with Tc-99m and injected i.v. into tumor xenograft-bearing mice, completing the task as planned. Mice were imaged and the $^{99m}$Tc AFP preparations were assessed by their resultant tumor to background ratios and clearance kinetics. Although we initially planned to focus on the best agent for further study (task 4), our initial results showed that both agents worked well and that there was not a clearly superior agent. We therefore proceeded to test both agents in xenografts of other tumors to characterize the relative performance of the agents. This task is complete (see attached manuscript).

### Task 4: Months 5-24: Compare the imaging capability of $^{99m}$Tc AFP to that of $^{99m}$Tc sestamibi and $^{201}$ thallium

Each tumor line was expanded in culture and then transplanted into SCID mice. Replicate tumor-bearing mice are imaged in random order with $^{99m}$Tc AFP, $^{99m}$Tc sestamibi, and $^{201}$ thallium . Biodistribution kinetics and tumor to background ratios for each test agent is assessed. Studies are repeated for a variety of tumors.

The information from the preliminary studies we have carried out suggests that Tc-99m AFP will be an excellent imaging agent for human breast cancer. The studies of aim 1 were designed to allow us to compare the candidate Tc-99m AFP molecules in two human breast cancer xenografts (MCF7 an estrogen receptor positive (ER+) tumor and MDA MB 231, an ER- tumor cell line). The purpose of this aim was to identify which of the available $^{99m}$Tc AFP molecules had the best tumor localization and imaging performance. This compound was to then be further tested in comparison with $^{99m}$Tc Sestamibi and $^{201}$Tl in a broad range of tumors. Image examples of the localization of the radiopharmaceuticals are shown in figure 2 of the attached manuscript. The quantitative comparisons of tumor percent injected dose/gram (%ID/gram) and tumor to background (T/B) are shown in the appendix. The results of the studies have been summarized using standard descriptive statistics. Comparison of differences between the different preparations have also been analyzed using analysis of variance with pair-wise comparisons done by Student-Newman-Keuls method.

We initially planned to compare $^{99m}$Tc radiolabeled natural full length AFP, recombinant full length AFP and recombinant domain III of AFP (DIII). Unfortunately, the supply of recombinant full length AFP from Atlantic Biopharmaceuticals was interrupted by financial instability and subsequent failure of that corporation. Our studies with the two remaining forms of AFP showed better performance of full length natural AFP in the MCF7 xenografts whereas the DIII radiopharmaceutical appeared superior in the MDA MB 231 xenografts (see appendix). Given the reduced number of candi-

date AFP compounds and the uncertainty as to which of the remaining would prove to be superior, we decided to evaluate both the full length AFP and DIII in all of the tumors studied.

We evaluated the $^{99m}$Tc AFP preparations by comparison to two other clinically available tracers that are being evaluated in patients with breast cancer: $^{99m}$Tc sestamibi and $^{201}$Tl. The tumors tissues represent breast and non-breast cancers that were steroid receptor positive or steroid receptor negative. Replicate tumor xenograft-bearing mice were used to assess the imaging capability of $^{99m}$Tc AFP $^{99m}$Tc sestamibi, and $^{201}$Tl for each tumor. Four imaging studies were performed in a set of six replicate tumor xenograft-bearing mice, one for each tracer. The studies were spaced by 3-5 days to allow the previous tracer to decay and to allow the animals to fully re-equilibrate. The sequence of tracer administration was random. The tumor size and appearance was recorded over the interval to correlate size with detectability and target to background ratio.

The data were analyzed to address three specific questions. 1] Is radioactivity in tumor significantly above that in background tissue? 2] Is radioactivity in tumor from $^{99m}$Tc AFP significantly above that from $^{99m}$Tc sestamibi or $^{201}$Tl? 3] Are there differences among tumors in radioactivity from $^{99m}$Tc AFP?

We have completed the analysis of the data and have found that the results support the hypothesis that AFP is concentrated in breast cancer cells, thereby providing a novel means for detecting breast cancer cells specifically and with high sensitivity. Moreover, the results indicate that $^{99m}$Tc AFP was a better imaging agent than $^{99m}$Tc sestamibi and $^{201}$thalium in all of the tumors studied (see appendix and manuscript-attached).

### Task 5: Months 5-10: Establish AFP receptor assay using MCF-7 and MDA-MB-231 breast cancer cell lines

We proposed to radiolabel AFP with 125I by the Chloramin T method and reproduce the studies reported by Uriel[18]. We then wished to evaluate the AFP receptor content of MCF-7 and MDA-MB-231.

An assay for AFP receptor was developed by our group. The published procedure using radioiodinated AFP utilizes prohibitively large amounts of cold AFP[18]. Therefore, a technique employing only radioinert AFP was needed. We took advantage of the exquisite sensitivity of the Abbott IM$\chi$ immuno-quantitation of AFP (0.2 ng/ml) and the published report that AFP is dissociated from its receptor in 0.4 M KCl. $2.5 \times 10^6$ cells in 0.2 ml serum-free medium were incubated with varying concentrations of AFP for 3 hours at 4°C. Cells were washed four times by centrifugation and resuspension in serum-free medium. After the final washing there was no detectable AFP in the supernatant. Sodium azide (20 mM, 5 min, 4°C) was then added to prevent receptor-ligand complexes from internalizing when cells were subsequently warmed. KCl (0.4 M final concentration) was then added and incubated for one hour at 37°C. Cells were then centrifuged at 2,000 rpm for 10 minutes, supernatant is removed and AFP content in the supernatant was determined. By Scatchard plot analysis of AFP bound at different incubation concentrations, the number of specific binding sites per cell and their binding affinity were determined. Binding of AFP plateaus at 30 ng/ml. Bound AFP was approximately 0.1% of total AFP added to the cells. Therefore, concentration of free AFP was assumed to be equal to that of total AFP. The overall purpose of the task has been achieved albeit by a different route than originally planned. This task is therefore considered complete.

### Task 6: Months 5-24: Establish AFP receptor content in various tumors

The method previously described by Uriel et. al.[18]. Briefly, studies are performed in duplicate, in siliconized 1.5 ml Eppendorf tubes containing $1 \times 10^6$ tumor cells and a tagged probe incubated in

0.2 ml serum-free RPMI medium. Incubations are terminated by centrifugation and removal of medium, and recentrifugation. Tube tips containing pelleted cells are severed and dropped into culture tubes for radioactivity estimation in a gamma counter. To com-

**Table 2: AFP Receptor Binding Study Results**

| TUMOR (P:prostate, B: breast) | MDA **B / ER-** | MCF 7 B / ER+ | T 47 B / ER+ | LNCaP P / AR+ |
|---|---|---|---|---|
| AFP Binding Kd | $6 \times 10^{-8}$ | $3.8 \times 10^{-8}$ | $1.8 \times 10^{-10}$ | $3.9 \times 10^{-9}$ |
| Receptors - #/cell | 140,000 | 43,800 | 30,200 | 31,000 |

pensate for possible loss of cells during the washings, the counted pellets are solubilized by addition of 0.5ml 0.1 N NaOH to the culture tubes, and an aliquot is assayed for protein by Bradford assay (Bio-Rad). This is compared to the protein content found after solubilizing $1 \times 10^6$ cells, and radioactivity per $10^6$ cells is thus computed.

The data yielded represent specific plus non-specific binding of probe to cells. Non-specific binding is estimated by performing identical incubations to these in the presence of a 50- to 100-fold excess of radioinert probe.

The data indicate high-affinity binding of AFP to human cancer cells. This was true of prostate as well as breast cancer cells, regardless of whether breast cancer cells were positive or negative for estrogen receptor. The data are consistent with our imaging studies, which showed good uptake of AFP into all of the tumor xenografts that were evaluated. The data are also consistent with earlier studies, which have suggested that AFP receptor is expressed in dedifferentiated cells [18-20]. Related studies by my collaborator, Dr. Bennett, have shown that AFP inhibits the growth of estrogen-receptor-positive, but not estrogen-receptor-negative, human breast cancer xenografts (Clin Cancer Res 4:2877-2884, 1998). Based on the imaging data and receptor data reported herein, the mechanism of this growth inhibition by AFP does not appear to be related to the uptake of AFP by these tumors.

## Task 7: Months 12-24: Studies of 99mTc AFP in rat homograft model of metastasis.

To assess the ability of $^{99m}$Tc AFP to detect spontaneous metastases we evaluated in the transplantable 13762 NF rat mammary adenocarcinoma model, which reliably metastasizes to draining lymph nodes within two weeks after transplantation into syngeneic rats [21].

We obtained the 13762 NF rat mammary carcinoma cell line and transplanted it into the dorsal region of the upper neck in female Fischer rats. The kinetics of metastasis to draining brachial and axillary nodes by necropsy and histopathology studies was investigated but scatter activity from the injection site made determination of activity in the lymphnode region unresolvable. We therefore developed a metastasis model system in which the investigation of metastasis uptake could be determined that did not require localized injection or would be confounded by uptake in the primary.

The 13762 NF rat mammary cancer cell line was obtained and grown as a monolayer in culture. Cells were harvested from culture and injected subcutaneously in rats. Tumors were palpable within 3-5 days following injection of $5 \times 10^6$ cells in the shoulder region of syngeneic Fischer rats.

Tumor became lethal 16-20 days after tumor implantation. Imaging was carried out when tumors were between 1.0 and 3.0 cm. $^{99m}$Tc-AFP imaged this tumor quite well, yielding average tumor-to-background ratios of 3.9 and 4.3 at 1 and 22 hours, respectively. This compares, respectively, to a

T/B of 5.4 and 4.7 for $^{99m}$Tc -AFP in MCF-7 human breast cancer xeno-
grafts. Axillary node metastases were not detectable on necropsy until
day 9 after tumor implantation, and lung metastases were not detectable
until day 14 after tumor implantation. By these times the primary tumor
was so large that it would have masked the imaging of spontaneous me-
tastases. In order to circumvent this problem, an artificial model of lung
metastases was established by injecting tumor cells intravenously. Tu-
mor nodules were visible in the lung by day 9 after inoculation of 2 x 10$^6$
cells. Metastases are found only in the lung and are lethal by day 16 after
implantation. As illustrated in figure 1 (right) $^{99m}$Tc-AFP has imaged
lung nodules, with one area of the lung particularly intense on the imag-
ing profile (circled). This area was hard on palpation during necropsy,
and, on further examination, was found to contain a dense tumor nodule
deep in the lung parenchyma. We expect that the task will be completed
as planned over the next 12 months.

## KEY RESEARCH ACCOMPLISHMENTS

Current methods of detecting breast cancer have low specificity
and sensitivity. Although screening mammography results in early de-
tection of breast cancer and reduces death from this disease, it has a low
positive predictive value and a 60-90% false positive rate that leads to
the pain, morbidity, and potential disfigurement associated with an esti-
mated 500,000 unnecessary breast biopsies. Furthermore, true positive
mammography is not helpful in assessing prognosis or predicting thera-

Figure 1. 24 hour image of rat with a lung metastasis visible in apical region of right lung (circle). Smaller nodules are evident in the mid lung region (arrow)

peutic response. Scintigraphic methods to detect breast cancer offer a means to improve the evalua-
tion of patients with positive breast exams or positive mammograms.

Alpha-fetoprotein is a serum protein produced by fetal liver and crosses into the maternal circula-
tion during pregnancy. Although developing tissues have the ability to bind and endocytose AFP,
this function is lost by adult differentiated cells [18-20]. The capability reappears, however, in neoplas-
tic cells growing either *in vivo* [22] or *in vitro* [18,23,24]. Malignant cells that have been shown to take
up AFP include human breast cancer cells, malignant lymphoblastoid cells, neuroblastoma cells, and
rhabdomyosarcoma cells [18,25,26]. Our collaborative "AFP group" has studied AFP and Domain III
of human AFP because of their properties as inhibitors of breast cancer growth.[7,27-30] We have
found that $^{99m}$Tc radiolabeled recombinant human AFP ($^{99m}$Tc AFP) localizes rapidly and specifically
in human breast cancer xenografts, providing well-defined images of the tumor relative to normal tis-
sues.[31] Our studies indicate that $^{99m}$Tc radiolabeled human AFP and domain III of human AFP show
substantially better localization in human breast tumor xenografts than either $^{201}$Tl or $^{99m}$Tc ses-
tamibi.[31,32] Furthermore, $^{99m}$Tc AFP localizes in both estrogen receptor positive (ER+) MCF-7 and
ER- MDA MB 231 breast tumors and has blood clearance through the kidneys. These features are
critically important to an agent that must have high specificity.

Although scintimammography using non-specific tracers such as $^{99m}$Tc sestamibi can improve the
specificity of diagnostic imaging, it is likely that further gains in both specificity and sensitivity can
be achieved with AFP because it is selectively taken up by breast cancer and has low non-specific
uptake in normal tissues. The fact that our results indicate that $^{99m}$Tc AFP demonstrates greater image

specificity for breast cancer than either $^{201}$Tl or $^{99m}$Tc sestamibi suggests strongly that $^{99m}$Tc AFP should be developed further for this purpose.

Tc-99m radiolabeled natural human AFP and the recombinant domain III of human AFP both have low non-specific tissue uptake and rapid renal clearance from blood. Localization of Tc-99m AFP in human breast cancer xenografts is initially rapid, increases with time, and is superior to Tc-99m sestamibi and Tl-201. Given its specific uptake by breast cancer cells, its low non-tumor localization and its rapid renal excretion, these Tc-99m AFP preparations are potential diagnostic agents for human breast carcinoma.

## REPORTABLE OUTCOMES

Published Abstracts of Work:

Line BR, Bennett JA, Jacobson H, Andersen T, Feustel P, Dansereau RN. Scintigraphic Imaging of Human Breast and Prostate Cancer Xenografts with Tc-99m Labeled Alpha-Fetoprotein. J. Nucl. Med. 1998; 39:222P.

Line BR, Dansereau RN, Andersen TT, Lukasiewicz RL, Bennett JA. Comparison of Tc-99m Human Alphafetoprotein to Tc-99m Sestamibi and Tl-201 in the Detection of Human Breast Cancer Xenografts. J. Nucl. Med. 1999; 40: 231P.

Submitted Manuscript:

Line BR, Feustel P, Festin S, Andersen TT, Dansereau RN, Lukasiewicz RL, Zhu SJ, and Bennett JA. Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein. J Nuclear Med (submitted 1999).

## SUMMARY & CONCLUSIONS

Current methods of detecting breast and prostate cancer have low specificity and sensitivity. Receptors for AFP have been detected on many malignant cells but are not expressed by normal tissue cells. We studied the binding affinity of AFP for human breast and prostate cancer cell lines (Scatchard Analysis) and evaluated the imaging characteristics of Tc-99m full length AFP (AFP) and Tc-99m recombinant human AFP Domain III (DIII) relative to Tc-99m sestamibi and Tl201. Studies were carried out using human tumor cell lines from estrogen receptor (ER) positive (MCF7, T47) and ER negative (MDA-MB231) human breast cancers as well as androgen receptor (AR) positive (LNCaP) and AR negative (DU 145) prostate cancers. Tumor xenografts were placed in the lateral thorax region of CB-17 SCID mice and grown to a size of approximately 0.8-2.0 cm diameter (0.27-4 gm). Tracer kinetics were measured at 0-60 minutes and at 24 hours following injection of 37MBq of either Tc-99m AFP (4-6 µg), Tc-99m DIII (4-6 µg), Tc-99m sestamibi or Tl-201.

Our results suggest that breast and prostate tumors demonstrate high affinity receptors for AFP. Tc-99m AFP and Tc-99m DIII specifically localize in human breast and prostate cancers in vivo with higher target to background localization than either Tc-99m sestamibi or Tl-201. Tc-99m AFP and Tc-99m DIII show promise as imaging agents for the detection and staging of breast and prostate cancer.

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## APPENDICES

Table 3A: Percent Injected Dose per Gram Tumor at 1 hour

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 0.98 (0.80) | [4] 2.30 (1.62) | [7] 0.12 (0.08) | [7] 0.33 (0.40) |
| DU145 | [8] 1.16 (0.64) | [3] 1.08 (0.15) | [5] 0.18 (0.16) | [4] 0.48 (0.29) |
| LNCaP | [10] 3.60 (2.48) | [5] 1.46 (0.09) | [5] 0.49 (0.29) | [4] 0.45 (0.08) |
| MCF7 | [2] 2.25 (0.22) | [2] 0.38 (0.06) | [5] 0.22 (0.14) | [6] 0.36 (0.27) |
| MDA | [6] 0.27 (0.09) | [10] 0.62 (0.31) | [6] 0.17 (0.09) | [6] 0.21 (0.15) |
| MFE | [5] 1.11 (0.54) | [5] 2.96 (2.28) | [4] 0.39 (0.19) | [6] 0.34 (0.16) |
| MTW9A | [5] 2.64 (1.16) | [4] 2.79 (0.30) | [3] 1.31 (0.37) | [5] 1.63 (0.82) |
| T47 | [2] 0.39 (0.85) | [8] 0.39 (0.25) | [2] 0.14 (0.13) | [3] 0.34 (0.18) |

Table 3B: Percent Injected Dose per Gram Tumor 24 hrs

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 0.44 (0.43) | [4] 1.23 (1.11) | [7] 0.01 (0.01) | [7] 0.06 (0.04) |
| DU145 | [8] 2.25 (2.80) | [3] 0.77 (0.67) | [5] 0.06 (0.04) | [4] 0.32 (0.29) |
| LNCaP | [7] 0.80 (0.48) | [5] 0.93 (0.30) | [5] 0.20 (0.19) | [4] 0.17 (0.13) |
| MCF7 | [2] 2.00 (1.89) | [2] 0.29 (0.16) | [2] 0.03 (0.01) | [5] 0.18 (0.18) |
| MDA | [6] 0.19 (0.10) | [12] 0.28 (0.16) | [6] 0.01 (0.01) | [6] 0.21 (0.06) |
| MFE | [5] 1.42 (0.64) | [5] 1.86 (1.52) | [4] 0.12 (0.08) | [6] 0.51 (0.11) |
| MTW9A | [5] 0.81 (0.58) | [4] 1.75 (0.38) | [3] 0.24 (0.08) | [4] 1.49 (0.19) |
| T47 | [2] 0.29 (0.06) | [8] 3.07 (2.35) | [2] 0.07 (0.00) | [3] 0.04 (0.03) |

Table 3C: Tumor to Thigh Tissue Background Ratio 1 hour

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 1.30 (1.28) | [4] 3.59 (2.54) | [7] 0.28 (0.26) | [7] 0.46 (0.53) |
| DU145 | [8] 1.33 (0.58) | [3] 3.00 (1.60) | [5] 0.13 (0.12) | [4] 0.22 (0.13) |
| LNCaP | [10] 4.32 (1.77) | [5] 2.73 (0.28) | [5] 0.99 (0.61) | [4] 0.38 (0.05) |
| MCF7 | [2] 4.10 (0.01) | [2] 0.55 (0.01) | [5] 0.29 (0.23) | [6] 0.28 (0.22) |
| MDA | [6] 0.56 (0.20) | [10] 1.57 (1.31) | [6] 0.41 (0.20) | [6] 0.15 (0.11) |
| MFE | [5] 2.60 (1.09) | [5] 8.50 (6.02) | [4] 0.65 (0.30) | [6] 0.49 (0.22) |
| MTW9A | [5] 2.95 (0.94) | [4] 4.62 (0.60) | [3] 0.81 (0.22) | [5] 0.65 (0.33) |
| T47 | [2] 0.30 (0.78) | [8] 0.78 (0.47) | [2] 0.11 (0.09) | [3] 0.34 (0.21) |

Table 3D: Tumor to Thigh Tissue Background Ratio 24 hrs

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 2.26 (2.18) | [4] 4.13 (3.85) | [7] 0.05 (0.07) | [7] 0.09 (0.06) |
| DU145 | [8] 1.65 (1.43) | [3] 1.65 (1.22) | [5] 0.10 (0.06) | [4] 0.26 (0.24) |
| LNCaP | [7] 3.79 (2.70) | [5] 4.44 (0.82) | [5] 0.97 (0.95) | [4] 0.19 (0.13) |
| MCF7 | [2] 4.56 (2.71) | [2] 1.28 (0.68) | [2] 0.09 (0.04) | [5] 0.20 (0.20) |
| MDA | [6] 0.93 (0.61) | [12] 1.17 (1.00) | [6] 0.10 (0.08) | [6] 0.23 (0.08) |
| MFE | [5] 6.04 (1.66) | [5] 10.93 (5.58) | [4] 0.65 (0.39) | [6] 0.61 (0.11) |
| MTW9A | [5] 3.21 (1.45) | [4] 3.87 (0.75) | [3] 0.63 (0.26) | [4] 0.87 (0.17) |
| T47 | [2] 0.86 (0.49) | [8] 4.62 (2.54) | [2] 0.15 (0.01) | [3] 0.03 (0.02) |

Data shown as number of animals in brackets, mean and standard deviation in parentheses (see description in body of report)

# Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein

Bruce R. Line, M.D.*, Paul J. Feustel, Ph.D.[†], Steven Festin, Ph.D.[¶], Thomas T. Andersen, Ph.D.[¶], Raymond N. Dansereau, Ph.D.[§], Roberta L. Lukasiewicz, B.S.*, ShuJi Zhu, M.D.[†], and James A. Bennett, Ph.D.[†]

Departments of *Radiology, [§]Pharmacy, [¶]Biochemistry and Microbiology and [†]Surgery
Albany Medical Center, Albany, NY

Address of Corresponding Author:
Bruce R. Line, M.D.
Professor of Radiology
Nuclear Medicine, A-72
Albany Medical Center
Albany, NY 12208

Tel: (518) 262-3361
Fax: (518) 262-3394
E-mail: bline@ccgateway.amc.edu

Reprint Requests to B. R. Line, M.D.

**Abbreviated Title Page:**


# Scintigraphic Detection of Breast Cancer Xenografts with Tc-99m Natural and Recombinant Human Alpha-Fetoprotein

## Abstract

Adenocarcinoma of the breast expresses receptors for alpha-fetoprotein (AFP). To reduce the number of patients with negative and potentially avoidable biopsy for mammographically suspicious lesions we have studied the potential of radiolabeled AFP to detect breast cancer scintigraphically. **Methods:** We radiolabeled natural (full length) and the recombinant domain III (DIII) of human AFP with Tc-99m (Tc-AFP) and compared their biodistribution with Tc-99m sestamibi and Tl-201 in a murine model of human breast cancer. Estrogen receptor positive (MCF-7, T47, MTW9A) and estrogen receptor negative (MDA MB-231, BT20) human breast cancer xenografts were grown subcutaneously to a size of 0.6 to 1 cm diameter (0.11-0.5 gm) in the lateral thorax region of immunosuppressed mice (CB-17 SCID). Tracer uptake was measured at 0-60 minutes and at 24 hours following injection of 37 MBq of Tc-AFP, Tc-99m sestamibi or Tl-201. Quantitative comparisons of percent-injected dose per gram of tissue (%ID/gram) and tumor to thigh ratio (T/Th) were performed for all tumors greater than 0.25 grams. **Results:** For most tumors, the T/Th ratios were greater than 1 for both AFP and DIII, whereas all of the ratios were less than 1 for Tc-99m sestamibi and Tl-201. For most tumors, T/Th for AFP and DIII was significantly greater than T/Th for Tc-99m sestamibi and Tl-201. There was no clear advantage of radiolabeled full length AFP over DIII AFP. In all breast cancers (BT20, MCF7, MDA, MTW9A, T47), T/Th increased from 1 to 24 hours with Tc-99m AFP suggesting good

tumor retention of this radiopharmaceutical. DIII and AFP had significantly

higher %ID/gram than Tl-201 or Tc-99m sestamibi when considered across all

tumor types at both 60 minutes and 24 hours. **Conclusion:** Tc-99m radiolabeled

natural human AFP and the recombinant DIII of human AFP both have low

non-specific tissue uptake and rapid renal clearance from blood. Localization

of Tc-99m AFP in human breast cancer xenografts is initially rapid, increases

with time, and is superior to Tc-99m sestamibi and Tl-201. Given its specific

uptake by breast cancer cells, its low non-tumor localization and its rapid renal

excretion, these Tc-99m AFP preparations are potential diagnostic agents for

human breast carcinoma.

## Introduction

Current methods of detecting breast cancer have low specificity and sensitivity. [1-3] Because of the lack of mammographic specificity, most biopsies of the breast are benign, with the probability of malignancy ranging from 15-35%.[4-7] The need for a test with greater specificity than mammography also arises in patients with dense breast tissue, in patients with prior breast surgery, and in those who are at higher risk for breast cancer for whom the mammogram is equivocal. Scintimammography is a widely available, non-invasive, and accurate imaging technique that may be used to further evaluate mammographically suspicious lesions and increase the specificity of breast cancer detection. For the scintigraphic agents having the best results to date (Tc-99m sestamibi, Tl-201, Tc-99m MDP, and F-18 FDG), tumor uptake is related to nonspecific factors such as metabolic rate, capillary permeability, and membrane ion transport. These small molecules also migrate into background tissues and reduce potential accuracy by clouding the scintigraphic image.

Alpha-fetoprotein (AFP) is a serum protein present in fetal tissue and in the maternal circulation during pregnancy, but is repressed at parturition. Certain dedifferentiated tissues actively take up AFP, presumably as part of a fatty acid transport system [8-11]. It is also taken up by certain cancer cell lines in vitro, including human breast cancer [12-14]. The affinity of AFP for human breast cancer cells in vitro and the lack of receptors in differentiated tissue suggest

that a radiolabeled molecule of AFP may be useful as a means to detect breast

cancer. Therefore, we studied the imaging potential of Tc-99m radiolabeled

human AFP and its recombinant DIII fragment in xenograft models of human

breast cancer in immune deficient mice and compared image data obtained with

these agents to that obtained with Tc-99m sestamibi and Tl-201.

## Materials and Methods

### Sources of AFP

### Preparation of Natural human full length AFP from human hepatoma

**(HepG2) cells.** HepG2 cells were maintained and grown as a monolayer in

αMEM (GIBCO, Grand Island, NY) supplemented with 5% serum (2/5 calf

serum, 3/5 fetal calf serum), penicillin G (100 units/ml), and streptomycin (100

μg/ml). Cells were released from monolayer using 0.25% trypsin/0.25% EDTA.

Subculturing into additional flasks was carried out by five-fold dilution of cells

in the above maintenance medium. Confluent flasks were switched to serum-

free medium to up-regulate production of AFP as described by Tecce et al. [15].

Serum-free medium was comprised of 3 parts αMEM: 1 part Waymouth's MB

752/1 plus $3 \times 10^{-8}$ M sodium selenite, 2 mM L-glutamine and 1.5%

antibiotic/antimycotic mixture from GIBCO (Grand Island, NY). Cells were

refed with serum-free medium every three days.

HepG2 culture supernatants were pooled and concentrated using P-10

Centriprep concentrators (Amicon, Beverly, MA). APF was purified from the

supernatant by loading ten ml of concentrate containing approximately 3 mg of

AFP onto an 18 cm x 2.5 cm immunoaffinity column (rabbit anti-human AFP

conjugated to cyanogen bromide-activated Sepharose 4B) in a loading buffer of

100 mM sodium chloride/10 mM sodium phosphate pH 7.4. Concentrate was

incubated on the column at room temperature for 30 minutes. Non-AFP

proteins were eluted with approximately 200 ml loading buffer until no protein

was detectable in the eluate by UV absorbence (280 nm). AFP was eluted with

approximately 200 ml of 1.8 M MgCl$_2$ and dialyzed immediately against excess

10mM sodium phosphate buffer, pH 7.4. This material was washed and

concentrated in a buffer comprised of 100 mM sodium chloride-10 mM sodium

phosphate, pH 7.4. The purified protein yielded a single band on silver-stained

gels, and the band was identified as AFP by Western blot using polyclonal

antibody to human full length AFP (Dako Corp., Carpinteria, CA)

**Recombinant Domain III AFP.** The third domain of AFP (DIII) was

produced using a baculovirus expression system. DIII was chosen because

previous studies had shown that the biological activity of AFP was localized to

third domain of the molecule [16,17] The cDNA encoding all of DIII and a small

part of domain II was obtained from the American Type Culture Collection

(Rockville, MD). The cDNA was amplified by polymerase chain reaction

(PCR) to yield a fragment that encoded for DIII only. This fragment was

subcloned in a TA cloning vector (Invitrogen, Inc.) in order to provide

convenient "sticky" ends (5'Xho I site and 3'Nsi I site). The subcloning step

permited directional cloning of the cDNA into a transfer vector (5'Xho I site

and 3' Nsi I compatible with Pst I "sticky" end) downstream of a polyhedron

promoter and flanked by viral sequences necessary for allelic replacement. The

transfer vector (pAcSGHisNT-B, Pharmingen, Inc.) was modified to include a

segment that codes for a polyHistidine region on the N-terminus of each DIII

AFP to facilitate purification by metal ion affinity chromatography.

PAcSGHisNT-DIII was inserted into modified Autographica californica nuclear polyhedrosis virus to generate a plasmid capable of producing a histidine-tagged DIII fusion protein. Plasmid was grown in Sf9 cells (Invitrogen, Inc.) and transferred to High Five cells (Invitrogen, Inc.) to obtain amplified protein expression of DIII AFP (including the polyHistidine linker at the N-terminus). After the protein was purified on an affinity column designed to bind the His linker (Talon resin, Clonetech, Inc.) and was eluted with imidazole, it yielded a single band on silver-stained gels. This band was identified as AFP by Western blot using polyclonal antibody to human full-length AFP (Dako Corp., Carpinteria, CA). Protein identity and quantity were further verified by amino acid analysis.

**Human breast cancer cell lines**

The MCF-7 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC), Rockville, MD, and were grown in DMEM supplemented with 5% fetal calf serum, 1% non-essential amino acids, 10 ng/ml insulin, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The BT20 human breast cancer cell line (ATCC) was grown in EMEM supplemented with 10% fetal calf serum, 1% non-essential amino acids and penicillin/streptomycin as above. The T47D human breast cancer cell line (ATCC) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml insulin, L-glutamine and penicillin/streptomycin as described above.

**Human breast cancer xenografts**

ICR severe combined immunodeficiency (SCID) female, five-week-old mice were purchased from Taconic Farms, Germantown, NY. Mice were housed in THORN+ units under laminar flow conditions and fed autoclavable mouse chow and water ad libitum. Human tumor pieces for transplantation into mice were obtained from cell lines that were released from monolayer by trypsinization, diluted into single-cell suspension and then solidified by centrifugation into a pellet. The pellet was encased in clot by exposure to 15 µl fibrinogen (50 mg/ml) (Sigma, St. Louis, MO) and 10 µl thrombin (50 units/ml) (Armour Pharmaceutical Company, Kankakee, IL) for 30 minutes at 37°C [18]. The fibrin clots containing tumor were cut into pieces approximately 1.5 mm in diameter. Six to eight pieces were loaded into a 16-gauge trocar and implanted subcutaneously in the lateral thoracic region of mice. Estrogen supplementation of mice was achieved by subcutaneous implantation of a Silastic tubing capsule containing solid estradiol (Sigma, St. Louis, MO) and was begun on the day of tumor implantation. Tumor growth was monitored by weekly measurement of tumor size using a vernier caliper. Tumors usually became palpable 3 weeks after implantation and attained a size of 1 cm in diameter 5 weeks after implantation. To assess tracer sensitivity, imaging studies were performed when tumors were approximately 0.6 cm in diameter, and as they enlarged to 1 cm in size. [18-20]

### Radiolabeling Procedure

Tc-99m labeling of AFP (full length and DIII) was carried out via stannous ion reduction as previously described [21]. Briefly, Tc99m AFP was prepared from a 50μg AFP aliquot mixed with 0.5ml 0.9% Sodium Chloride Injection (Baxter Healthcare). The solution was added to an Ultra Tag RBC™ Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO), and the contents of the vial were mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation time, 370-740 MBq Tc-99m Sodium Pertechnetate Injection obtained from a freshly eluted generator (Mallinckrodt Medical, Inc., St. Louis, MO) was added in a volume of 1-2 ml. The contents of the vial were mixed by gentle swirling and were incubated for 15 minutes. Dose aliquots were assayed for integrity of binding of Tc-99m to AFP using thin-layer chromatography. Typically, 92 - 100% of the Tc-99m was bound to AFP. The preparation was not used in studies if the percent bound was less than 90%. To define the stability of the Tc-99m AFP preparations, thin-layer chromatography in acetone (ITLC-SG, Gelman Instrument Co., Ann Arbor, MI) was again used to determine the degree of Tc-99m that was unbound with shelf storage for 3 and 6 hours at room temperature. Sestamibi was radiolabeled using kits for the preparation of Tc-99m Sestamibi Injection (DuPont Pharma, Inc).

**Tracer Biodistribution and Imaging Studies**

*In vivo* biodistribution data was collected in up to 6 mice imaged

simultaneously on a gamma camera. After sedation with intraperitoneal

administration of 50 mg/kg pentobarbital, the mice were injected intravenously

with 20-40 MBq of the tracer compound (Tc-99m AFP (3µg), Tc-99m

sestamibi, or Tl-201) and then were placed in the prone position on the gamma

camera collimator. To eliminate motion during imaging, the mice were

restrained by strips of tape over their extremities so as not to restrict respiration.

Twelve sequential, five minute images were obtained with low energy general

purpose collimation, and 1.5 fold hardware zoom into computer matrices

having 128 by 128 picture elements.[22] The animals were reanesthetized at 24

hours post injection and two thirty minute static images were obtained. The

data was transferred to a Pentium PC MS Windows-based image processing

system for analysis.[22,23].

The image data were evaluated to determine the activity in the tumor tissue

by drawing a region of interest that included all margins of the tumor. Where

the tumors were not scintigraphically visible, regions were placed over the area

where the tumor was palpated at the time of imaging. A second region was

placed over the contralateral chest wall by reflecting the tumor region of

interest through the midline axis. The second region was used to define a

background activity that was subtracted from the tumor region to yield counts

in the tumor tissue. The gram weight of the tumor was determined from the

tumor volume as calculated from the measured tumor diameter. A third region of interest was defined over the thigh and the counts per gram of thigh tissue was obtained by using a thigh mass of 6% of body weight. A fourth region of interest was defined over the entire animal to determine the total injected activity. Regions were produced for each animal study at both 60 minute and 1440 minute (24 hr) image collection points. Tracer localization parameters were determined by 1) the tumor uptake as the percent of injected activity per gram of tissue (%ID/gram); and 2) the tumor to thigh tissue background (T/Th) ratio determined from the ratio of tumor activity per gram to the thigh activity per gram.

**Statistical Analysis Methodology**

For each tumor, all four tracers were studied in the same six tumor bearing mice with a one-week rest period between the administration of each tracer. The order in which the tracers were injected for each tumor type was randomly chosen. Standard descriptive statistics (mean and standard deviation) were used to summarize the percent-injected dose/gram of tumor (%ID/gram) and tumor to thigh ratio (T/Th) results. Analysis of %ID/gram and T/Th ratios was by repeated measures analysis of variance (ANOVA) with fixed main effects of tumor type and tracer. Repeated measures analysis included a main effect of time (for the 60 and 1440-minute determinations) as well as interactions (tracer with tumor, tracer with time, tumor with time and tumor with tracer with time).

When justified by statistically significant main or interaction effects, multiple

comparisons were performed by the Student Newman Keuls multiple range test.

## Results

### *In-Vivo* Studies of Tc-99m AFP Uptake by Human Breast Cancer Xenografts

Using both Tc-99m radiolabeled preparations of full length human AFP and its recombinant DIII, SCID mice bearing either estrogen receptor positive (MCF-7, T47, MTW9A) or estrogen receptor negative (MDA MB-231, BT20) breast cancer xenografts were imaged continuously over one hour and then re-imaged at 24 hours. The mice showed little accumulation of tracer by any normal tissue other than kidney during the first hour after injection. By 24 hours, the tracer was cleared from both the blood and normal tissues and was found primarily in the bladder (Figure 1).

We compared the imaging capability of the Tc-99m AFP preparations to two other clinically available tracers that are being evaluated in patients with breast cancer: Tc-99m sestamibi and Tl-201. The tumor size and appearance was recorded over the interval to incorporate size into measures of detectability and tumor to thigh tissue ratio. Tumor size ranged from 0.5 to 2.0 cm in diameter. Examples of imaging studies are shown in figure 2.

The image data were analyzed to address three specific questions. 1) Is radioactivity in tumor significantly above that in background thigh tissue? 2) Is radioactivity in tumor from Tc-99m AFP significantly above that from Tc-99m sestamibi or Tl-201? 3) Are there differences among tumors in radioactivity from Tc-99m AFP? To evaluate whether the activity in tumor is significantly

greater than thigh tissue activity, we computed the ratio of counts per gram of tumor to the counts per gram of thigh tissue (T/Th). To compare levels of activity between tracers in a given tumor and for Tc-99m AFP preparations across tumors, the percent injected dose/gram of tumor (%ID/gram) was calculated. The quantitative comparisons of T/Th ratio and %ID/gram are shown in figures 3 and 4 and are detailed in Table 1, parts A-D. This evaluation was performed for all tumors greater than 0.25 grams. Tumors smaller than this were excluded because they were difficult to locate reliably in the images. In the T/Th results, values greater than one indicate higher tumor localization than thigh tissue activity. The tumor activity was compared to thigh background instead of the contralateral chest region, because, at the edge of the thorax, small changes in contralateral chest region placement produced highly variable target to background ratios.

In all tumors studied, the Tc-99m AFP preparations yielded a T/Th ratio that was greater than 1, while Tc-99m sestamibi and Tl-201 yielded a T/Th ratio that was less than 1. This was true both at 60 minutes and 24 hours after tracer administration.

When considering all tumor types together, ANOVA showed that DIII had significantly higher T/Th than AFP that, in turn, had significantly higher T/Th than both Tc-99m sestamibi or Tl-201. Because the tracers differed in their ability to detect different tumors (reflected as a significant interaction between tracer and tumor type) subsequent ANOVA with Student Newman Keuls

multiple comparison tests were performed for each of the tumor types.  Figure 3

shows 60 minute T/Th values which were generally lower than the 24 hour

T/Th values, although there was little overall difference in the comparisons

made at the two times.  In most comparisons the T/Th measures are

significantly greater for AFP and DIII relative to Tc-99m sestamibi and Tl-201

(see figure 3 for statistical significance of comparisons).

DIII and AFP had significantly higher percent injected dose per gram of

tumor tissue than Tl-201 or Tc-99m sestamibi ($p < 0.05$) when considered across

all tumor types (Figure 4).  This was true at both 60 and 24 hours after tracer

administration. There was no clear-cut difference in the imaging capability

between radiolabeled full length AFP and DIII AFP. For percent injected dose

per gram of tumor, all ANOVA main effects as well as the interaction of tumor

type with tracer and tumor type with time were statistically significant.  The

main effect of tracer was that DIII and AFP were greater than Tc-99m sestamibi

or Tl-201 ($P < 0.05$). Figure 4 shows 60-minute percent-injected dose per gram

of tumor, which were generally higher than the 24-hour values. When

considering individual tracers and tumors, %ID/gram for DIII was significantly

($p < 0.05$) higher than Tc-99m sestamibi or Tl-201 in BT20, MCF7, MDA, and

MFE tumors (Figure 4).  The %ID/gram for AFP showed a similar trend but

achieved statistical significance over Tc-99m sestamibi and Tl-201 only in the

MCF7 tumor and over Tc-99m sestamibi in the MTW9A tumor. Thus, Tc-99m

AFP and Tc-99m DIII appear to offer significant imaging advantages in a wide

range of tumor cell lines with regard to tumor uptake and relative contrast to

normal tissue.

## Discussion

The results of this study demonstrate that AFP (natural or recombinant) labels efficiently with Tc-99m and selectively localizes in human breast cancer xenograft tissue in vivo. One hour following i.v. administration of Tc-99m AFP, label is found primarily in the tumor and in the renal system. These results are in agreement with those reported by Uriel et al.[13], who showed selective localization of murine I-125 AFP in syngeneic mouse mammary cancers in vivo. The mechanism by which AFP localizes in cancer cells may be via an AFP receptor described by Villacampa et al.[12]. Malignant cells that have shown receptor-mediated uptake of AFP include human breast cancer cells, malignant lymphoblastoid cells, neuroblastoma cells, rhabdomyosarcoma cells and lymphoblastoid cells[12,13,24]. Although most dedifferentiated tissues have the ability to bind and endocytose AFP, this function is lost by differentiated cells. [12,25,26] The loss of this function in adult cells may be the basis for the selective tumor localization of AFP seen in this study. These studies are the first to show selective human tumor localization of Tc-99m radiolabeled human AFP. The imaging capability of AFP compares favorably to that of Tl-201 and Tc-99m sestamibi, agents currently used to detect breast cancer in humans. Although results with Tc-99m sestamibi and Tl-201 have shown promise,[27-29] improvements in both sensitivity and specificity are needed. In our study, Tc-99m AFP had substantially better imaging characteristics for human breast cancer xenografts than did either Tl-201 or Tc-99m sestamibi. This finding, coupled

with the fact that AFP is an endogenous human molecule with no known toxicity, strongly suggests that AFP should be developed further as an imaging agent for breast cancer. Both natural full-length AFP and DIII AFP were similar in their ability to image breast cancer in this study. For large-scale production, it seems reasonable to pursue recombinant systems and, in fact, such efforts are underway [30].

It is estimated that in the United States, 182,000 new cases of breast cancer will be diagnosed in women and 46,000 women will die of the disease [31]. Over 70% of U.S. women in the age groups at risk for breast cancer have had at least one mammogram, a critically important, but imperfect, screening study [1]. An abnormal mammogram will detect a mass, a cluster of calcifications, or both, but these findings are not diagnostic of breast cancer. If mammographic studies are abnormal, a breast biopsy is performed, or the abnormality is reassessed with periodic follow-up mammograms until the nature of the abnormality is determined [32].

Most biopsies of the breast are benign. The probability of malignancy when a biopsy is performed on a mammographic abnormality usually ranges from 15% to 35% [4-6,33]. Consequently, for every woman who has a breast cancer detected by screening mammography, three to six women with false-positive results needlessly undergo the apprehension, pain, and possible disfigurement associated with biopsy. The cost per surgical biopsy, including

preoperative and postoperative examinations, anesthesia, and hospitalization ranges between $3,000 and $5,000 [34].

In conclusion, scintigraphic methods that detect breast cancer offer a means to improve the evaluation of patients with positive breast exams or positive mammograms.  We have found that Tc-99m AFP images human breast cancer xenografts better than the clinically useful scintimammographic agents, Tl-201 and Tc-99m sestamibi.  Thus, it seems reasonable that Tc-99m AFP should be further developed as a radiopharmaceutical for the detection of human breast cancer.

## Acknowledgements

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## Tables

Table 1A: Tumor to Thigh Tissue Ratio at 1 hour*

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 1.30 (1.28) | [7] 3.35 (2.42) | [8] 0.24 (0.26) | [8] 0.55 (0.56) |
| MCF7 | [4] 5.41 (1.84) | [6] 2.83 (1.80) | [8] 0.45 (0.43) | [8] 0.19 (0.27) |
| MDA | [6] 0.56 (0.20) | [10] 1.57 (1.31) | [6] 0.41 (0.20) | [6] 0.15 (0.11) |
| MFE | [6] 3.54 (2.51) | [5] 8.50 (6.02) | [6] 0.71 (0.69) | [6] 0.49 (0.22) |
| MTW9A | [9] 3.58 (1.39) | [4] 4.62 (0.60) | [3] 0.81 (0.22) | [5] 0.65 (0.33) |
| T47 | [4] 0.37 (0.47) | [11] 1.18 (1.43) | [3] 0.03 (0.14) | [3] 0.34 (0.21) |

Table 1B: Tumor to Thigh Tissue Ratio at 24 hrs

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 2.26 (2.18) | [7] 4.07 (3.33) | [8] 0.08 (0.11) | [8] 0.16 (0.20) |
| MCF7 | [4] 4.70 (1.81) | [6] 5.33 (4.30) | [3] 0.26 (0.37) | [7] 0.21 (0.18) |
| MDA | [6] 0.93 (0.61) | [12] 1.17 (1.00) | [6] 0.10 (0.08) | [6] 0.23 (0.08) |
| MFE | [6] 6.28 (1.60) | [5] 10.93 (5.58) | [6] 0.79 (0.49) | [6] 0.61 (0.11) |
| MTW9A | [9] 5.96 (4.17) | [4] 3.87 (0.75) | [3] 0.63 (0.26) | [4] 0.87 (0.17) |
| T47 | [4] 1.80 (1.34) | [11] 3.90 (2.50) | [3] 0.27 (0.20) | [3] 0.03 (0.02) |

Table 1C: Percent Injected Dose per Gram of Tumor at 1 hour

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 0.98 (0.80) | [7] 2.24 (1.65) | [8] 0.11 (0.08) | [8] 0.43 (0.46) |
| MCF7 | [4] 3.54 (1.79) | [6] 1.79 (1.15) | [8] 0.46 (0.62) | [8] 0.24 (0.35) |
| MDA | [6] 0.27 (0.09) | [10] 0.62 (0.31) | [6] 0.17 (0.09) | [6] 0.21 (0.15) |
| MFE | [6] 1.47 (1.00) | [5] 2.96 (2.28) | [6] 0.42 (0.40) | [6] 0.34 (0.16) |
| MTW9A | [9] 2.70 (1.00) | [4] 2.79 (0.30) | [3] 1.31 (0.37) | [5] 1.63 (0.82) |
| T47 | [4] 0.37 (0.49) | [11] 0.63 (0.82) | [3] 0.04 (0.18) | [3] 0.34 (0.18) |

Table 1D: Percent Injected Dose per Gram of Tumor 24 hours

| Tumor | AFP | Domain III | Sestamibi | Thallium |
|---|---|---|---|---|
| BT20 | [8] 0.44 (0.43) | [7] 1.37 (1.14) | [8] 0.02 (0.05) | [8] 0.16 (0.27) |
| MCF7 | [4] 3.36 (2.09) | [6] 1.33 (0.89) | [3] 0.09 (0.14) | [7] 0.21 (0.20) |
| MDA | [6] 0.19 (0.10) | [12] 0.28 (0.16) | [6] 0.01 (0.01) | [6] 0.21 (0.06) |
| MFE | [6] 1.36 (0.59) | [5] 1.86 (1.52) | [6] 0.15 (0.09) | [6] 0.51 (0.11) |
| MTW9A | [9] 1.04 (0.61) | [4] 1.75 (0.38) | [3] 0.24 (0.08) | [4] 1.49 (0.19) |
| T47 | [4] 0.50 (0.30) | [11] 2.43 (2.26) | [3] 0.09 (0.04) | [3] 0.04 (0.03) |

*Data shown as number of animals in brackets, mean and standard deviation in parentheses

## Figure Legends

**Figure 1**. Biodistribution and clearance of Tc-99m full length AFP in a

SCID mouse bearing a 0.05 gram human breast cancer (T47) in the left axillary

region. The mouse was imaged with a very small tumor to assess biodistribution

with little interference from tumor. Images A, B, and C are obtained at 8, 60 and

1440 minutes (24 hours), respectively, after dose administration. Immediate

blood pool activity is evident in the heart region (solid arrow) which fades rapidly

and is nearly at background levels by 60 minutes. Persistent activity is evident in

the liver (dashed arrow) and kidneys at both 1 and 24 hours. No bowel activity is

evident and soft tissue activity is low at 1 and 24 hours. A slight asymmetry is

evident in the left axillary region at the site of the tumor, which is just palpable

(size<grape seed).

**Figure 2.** Images of human breast cancer xenografts in SCID mice bearing

1.0 to 1.5 cm diameter MCF7 tumor in the left axillary region obtained

respectively with Tc-99m AFP, Tc-99m DIII, Tc-99m sestamibi and Tl-201 at

weekly intervals in the same mouse (panels A-D respectively). Panels E-H show

the same tracers in 0.5 cm to 1.0 cm diameter BT20 human breast cancer

xenografts. All images are obtained at 24 hours after tracer administration.

**Figure 3.** Tumor to thigh ratios obtained at 60 minutes post injection.

Values are expressed based on the activity in a gram of tissue in the thigh and

tumor regions (see text for details). In general, the ratios were greater than 1 for

the AFP tracers and less than one for sestamibi and thallium. Analysis of

variance using the Student Newman Keuls multiple comparison test revealed

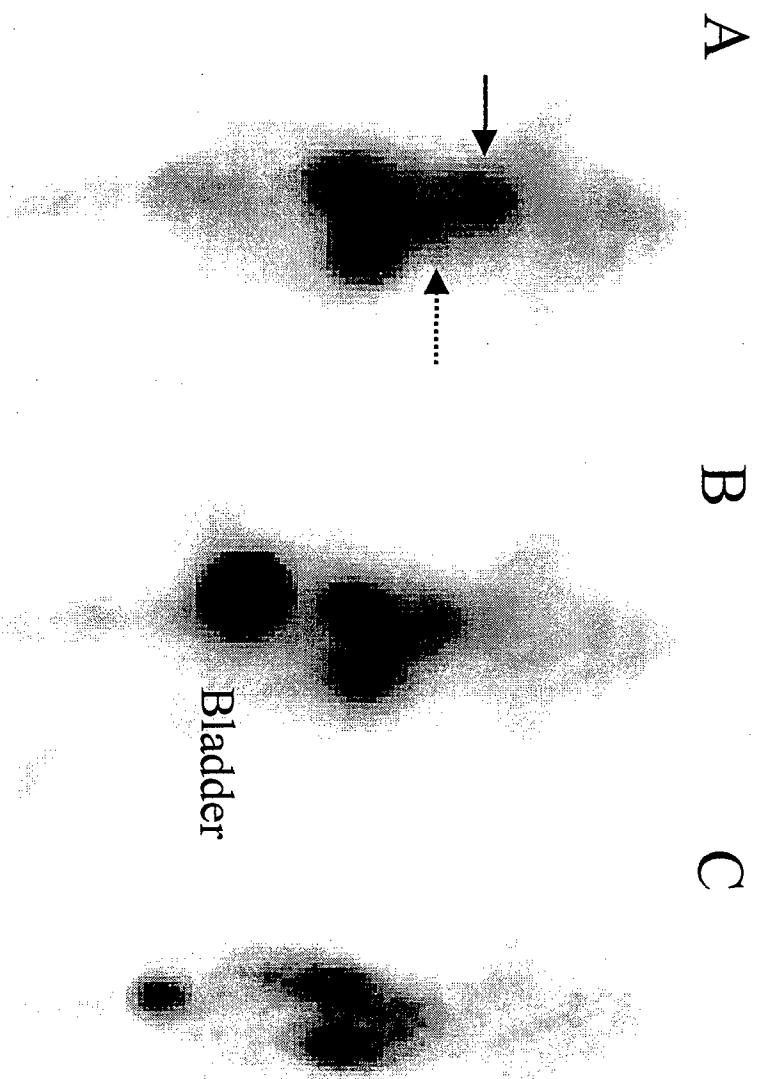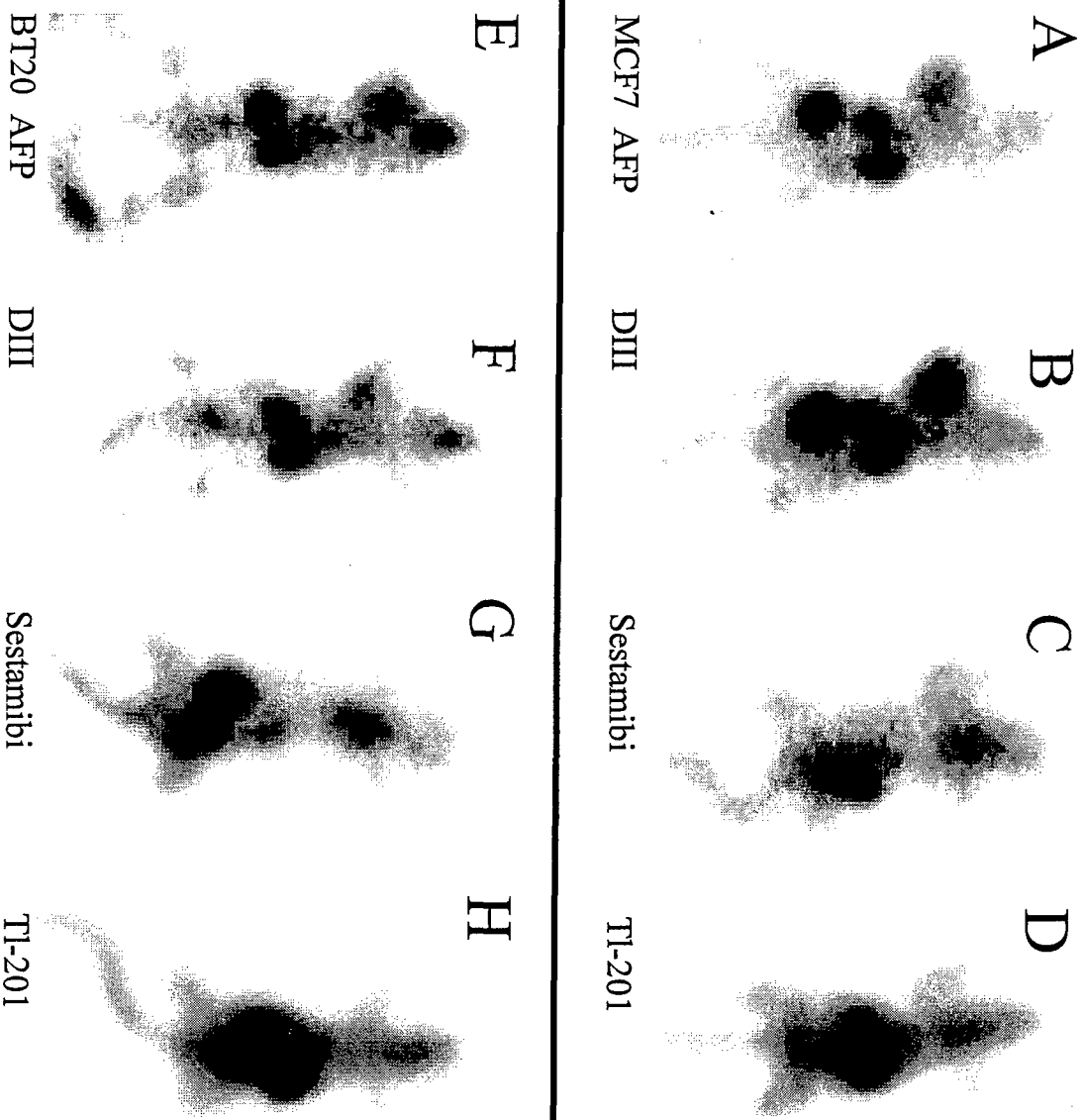significant differences compared to sestamibi (*) and compared to Tl-201 (#).

Figure 4. Percent injected dose per gram of tumor based on tumor weight,

tumor region activity and total activity in the mouse at 60 minutes post injection

(see text for details). Overall, values for Tc-99m AFP and Tc-99m DIII are

greater than Tc-99m sestamibi and Tl-201 in all tumors studied. Analysis of

variance using the Student Newman Keuls multiple comparison test revealed

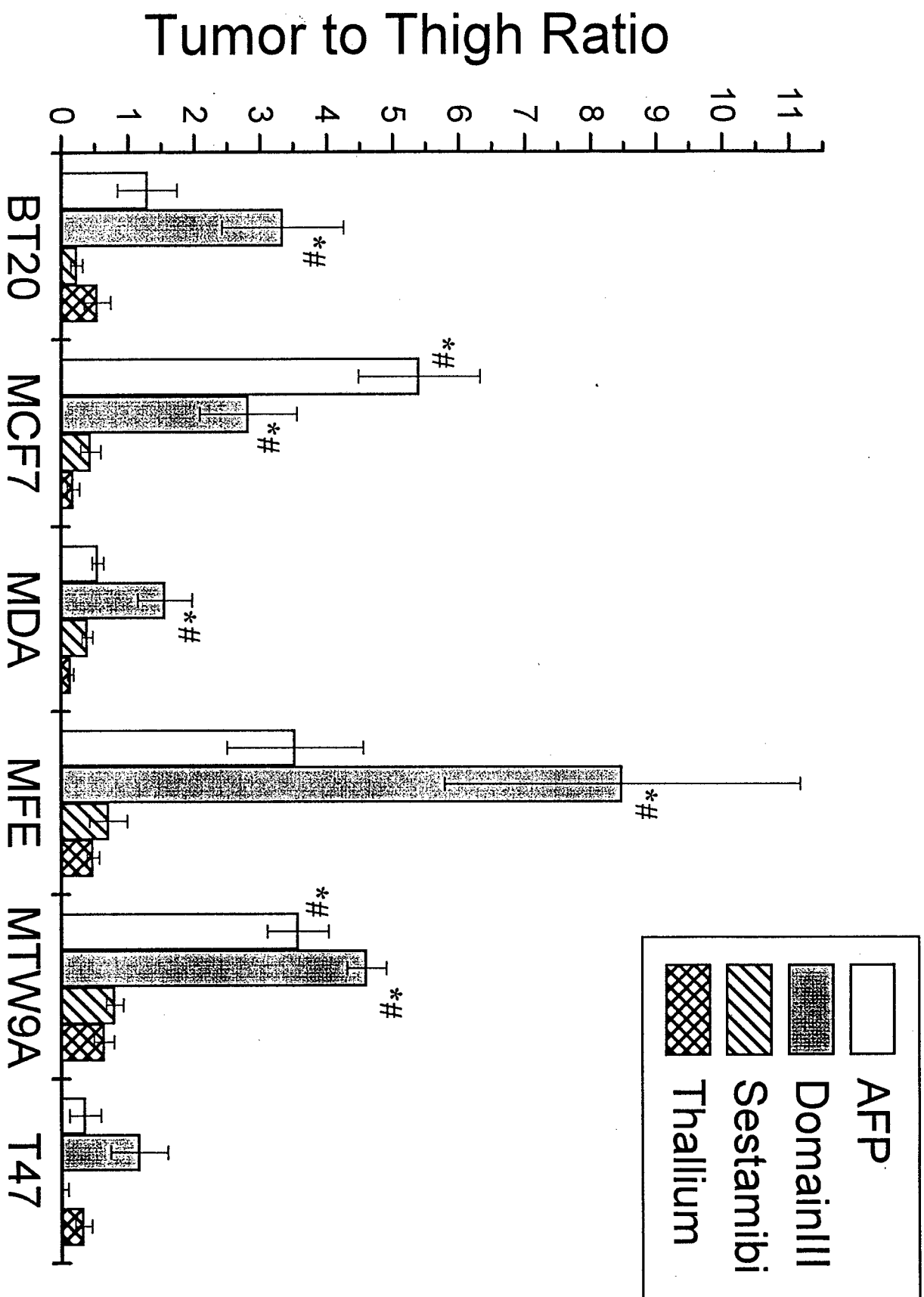significant differences compared to sestamibi (*) and compared to Tl-201 (#).

A    B    C

Bladder

Figure 1

| A | B | C | D |
| MCF7 AFP | DIII | Sestamibi | Tl-201 |

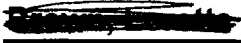| E | F | G | H |
| BT20 AFP | DIII | Sestamibi | Tl-201 |

Figure 2

Figure 3

**From:**     Miller, Virginia M Ms USAMRMC [Virginia.Miller@DET.AMEDD.ARMY.MIL]
**Sent:**     Tuesday, May 16, 2000 10:49 AM
**To:**       'lbrown@dtic.mil'
**Subject:**  Change to ADA376180

Hi Loretta,

Here is another report that was submitted as a Final before we knew of an
extension for ADA376180, Contract No. DAMD17-97-1-7145. Could you please
change the Descriptive Note to the following on the citation in the
database, cover and 298:

      Annual rept. 15 Jun 98 - 14 Jun 99.

Do you need to have a new cover and 298 faxed to you? Thanks again.

Virginia


Virginia Miller
Technical Information Specialist
USAMRMC, MCMR-RMI-S
301-619-7327, FAX: 301-619-2745
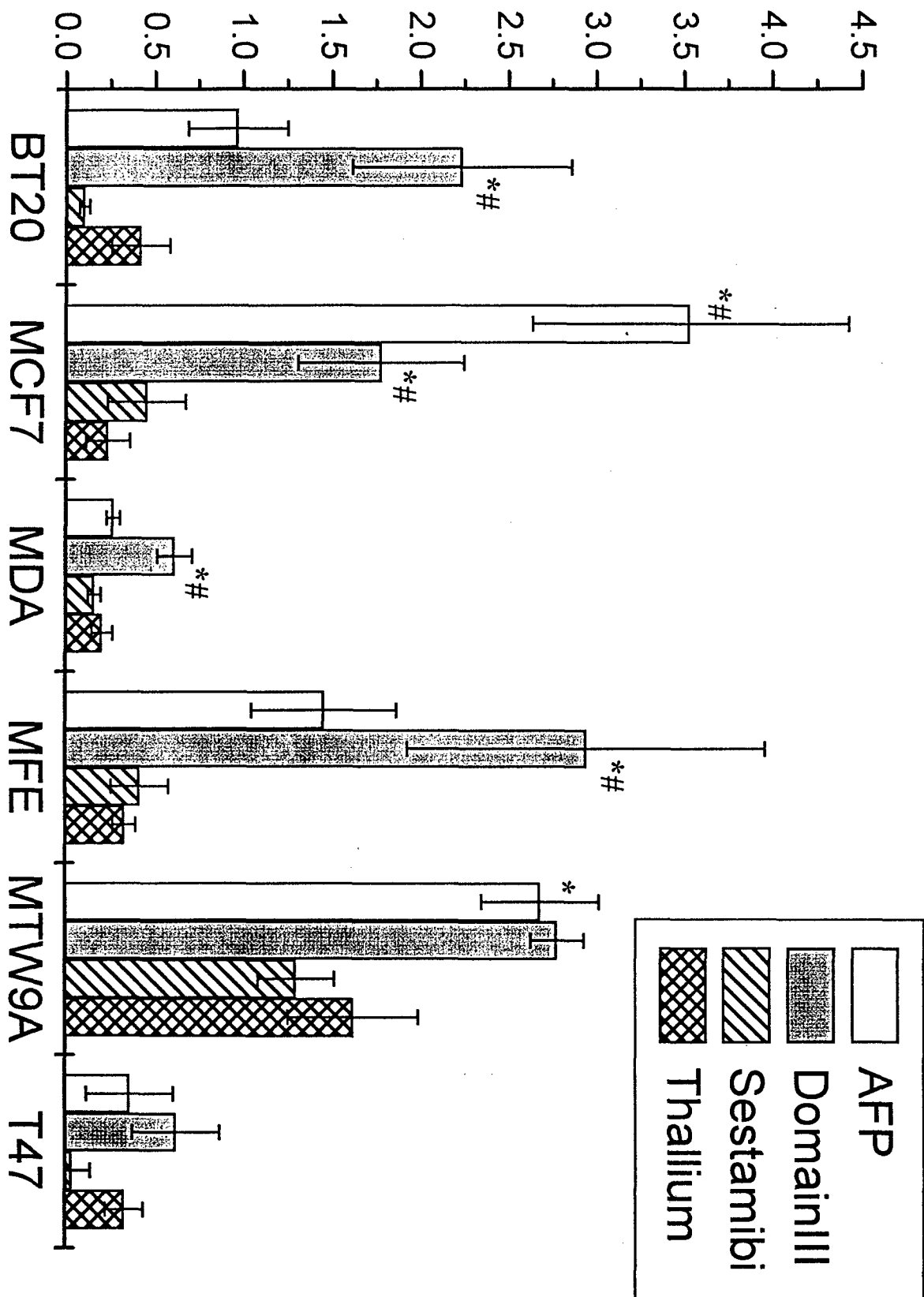virginia.miller@det.amedd.army.mil

% Injected Dose / gram of tumor

Figure 4